

1 *Regular paper*

2 **Title**

3 Understanding genetic diversity of relict forests. Linking long-term isolation legacies and
4 current habitat fragmentation in *Abies pinsapo* Boiss.

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Accepted refereed manuscript of:

Cobo-Simón I, Méndez-Cea B, Jump A, Seco J, Gallego F & Linares JC (2020) Understanding genetic diversity of relict forests. Linking long-term isolation legacies and current habitat fragmentation in *Abies pinsapo* Boiss. *Forest Ecology and Management*, 461, Art. No.: 117947.

DOI: <https://doi.org/10.1016/j.foreco.2020.117947>

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17 **Abstract**

18 Increasing variability and uncertainty regarding future climate provide new challenges for the conservation of
19 endangered tree species. For example, threat status can be impacted by genetic diversity, where forest trees
20 show reduced geographic range size, isolated populations and fragmented distribution. We place the
21 conservation insights of population genetic structure in a climate change context, using as experimental
22 system a relict drought-sensitive fir (*Abies pinsapo* Boiss.). Nuclear (nSSR, ISSR) and chloroplast (cpSSR)
23 markers were analysed to investigate the extent to that *A. pinsapo* evidences ongoing genetic erosion, isolation
24 and divergent genetic diversity, among populations, elevations and cohorts (young, adult and old trees). We
25 obtained contrasting patterns among chloroplast and nuclear markers. Based on cpSSRs, the highest genetic
26 distances were found in the western portion of the distribution, while based on both nSSRs and ISSRs,
27 differentiation appeared in the eastern portion of the distribution. Evidence for bottlenecks and genetic drift
28 were found in all the studied populations, as well as low among-population genetic differentiation. Land use
29 legacies e.g. impacting current forest structural diversity might be related to observed genetic diversity. No
30 evidence of demographic genetic erosion among cohorts was found. Conservation efforts should focus on
31 reducing the probability of occurrence of stochastic events such as fires and habitat loss due to human impacts
32 or climate change to maximise *A. pinsapo* population sizes. Further research on adaptive potential should
33 focus on identifying active genetic management strategies that might improve adaptation to future climates in
34 such endangered relict species.

35 **Keywords:** Gen flow; Genetic diversity; Genetic drift; Inbreeding; Adaptive management;
36 Circum-mediterranean firs; Conservation genetics; Microsatellite marker.

37

38 **Highlights**

- 39 • *Abies pinsapo* maintained relatively high genetic diversity.
- 40 • *A. pinsapo* shows low among-population genetic differentiation.
- 41 • Local climate and land-use legacies were related to haplotype diversity.
- 42 • Nuclear and chloroplast markers provide contrasting patterns of genetic diversity.
- 43 • Bottlenecks and genetic drift evidences were found in all the studied populations.
- 44 • Genetic erosion was not observed among cohorts of saplings, mature and old trees.
- 45

46 **1. Introduction**

47 High variability and uncertainty regarding potential future climates provides new challenges for the
48 adaptive management of drought-sensitive forest ecosystems (Jump and Peñuelas 2005; Aitken et al., 2008;
49 Alberto et al., 2013). The threat status of several species relies to some extent on genetic diversity, for example,
50 in forest trees with reduced geographic range size, isolated populations and fragmented distribution (Fady and
51 Conord 2010; Hampe and Jump 2011; Rehm et al., 2015). Tree species, as long-lived and sessile organisms,
52 mainly depend on their current genetic variation to develop locally-adapted phenotypes (Petit and Hampe
53 2006). Consequently, understanding evolutionary consequences of global climate change and its long-term
54 effects on biodiversity requires the investigation of the effect of range size, geographic isolation and
55 fragmentation on intraspecific genetic diversity (Kuparinen et al., 2010; Franks and Hoffmann 2012; Alberto et
56 al., 2013).

57 The practical application of the theories underlying adaptive capacity and genetic diversity to the
58 management of relict populations is a main concern regarding conservation biology (Neale and Wheeler 2019).
59 Here, well-known patterns, such as the relationship between heterozygosity and population fitness, support the
60 need to assess the processes of genetic erosion, genetic drift, or inbreeding to conserve genetic diversity (Reed
61 and Frankham 2003). Furthermore, exploration of the limitation of gene flow due to isolation and small
62 population sizes provides the basis to understand the current spatial genetic structure and to define reliable
63 conservation strategies aimed to reduce the loss of genetic diversity (Ledig et al., 1997, 2002; Jaramillo-Correa
64 et al., 2006, 2008; Eliades et al., 2011; Aleksić and Geburek 2013; Awad et al., 2014).

65 Reduced gene flow and distinct ecotypes are expected in remnant populations (Kremer et al., 2012). Thus,
66 quantification of the genetic diversity among populations of relict species is required to improve conservation
67 planning (Neale and Wheeler 2019). Furthermore, reliable spatial and temporal inference of recent genetic and
68 demographic changes may contribute towards a better understanding of the evolutionary process accounting for
69 sometimes high levels of genetic diversity in relict species confined to climatic refugia (Hampe and Jump
70 2011). Range size plays here an essential role in shifting patterns of genetic diversity (Hampe and Petit 2005).
71 Specifically, low genetic variation but high genetic differentiation is theoretically expected in relict populations
72 located in fragmented landscapes (Hampe and Jump 2011; Rehm et al., 2015). Notwithstanding, it has been
73 reported that endemic species, usually with restricted geographic ranges, may also show high genetic diversity
74 (Ledig et al., 1997, 2002; Eliades et al., 2011; Aleksić and Geburek 2013).

75 Biogeography has an important effect on genetic diversity and structure. For instance, the conifers from
76 the Mediterranean basin show higher genetic diversity, compared to those from other regions (Fady-Welteren,

77 2005). Furthermore, decreasing genetic diversity has also been recognized within populations of several taxa in
78 the western locations across the Mediterranean Basin (Fady and Conord 2010). Hence, Mediterranean relict
79 forests provide suitable experimental systems to investigate the effect of range size, geographic isolation and
80 fragmentation in shaping patterns of genetic diversity (Hampe and Jump 2011). These biogeographic and
81 evolutionary characteristics enhance the need to design adaptive management guidelines aimed to preserve
82 Mediterranean relict forests (Hampe and Petit 2005; Hampe and Jump 2011; Rehm et al., 2015).

83 Among them, some taxa seem to be particularly vulnerable to the current changing climate, as might be the
84 case of the relict circum-mediterranean firs (*Abies* Mill.), tree species that are, in many cases, near to their
85 tolerance limits, and therefore might be considered among the most sensitive ecosystems to current climate
86 change (Sánchez-Salguero et al., 2017). The current genetic diversity of the circum-mediterranean firs has been
87 recognized to a large degree as a consequence of ice age isolation in southern refugia and postglacial
88 colonization northwards (Linares, 2011), while these phylogeographical patterns may be currently constraining
89 the adaptive capacity of those remnant fir forests (Fady and Conord 2010; Liepelt et al., 2010). Furthermore,
90 land-use changes that have occurred during the last decades represent an additional predisposing factor to
91 climate-induced decline and mortality for several Mediterranean fir forests (Linares et al., 2009; Lechuga et al.,
92 2017, 2019; Alba-Sánchez et al., 2019).

93 Here we place the conservation insights of population genetic structure in a climate change context, using
94 as an experimental system the relict drought-sensitive fir *Abies pinsapo* Boiss. To date, it is not known whether
95 *A. pinsapo* lost genetic diversity following Holocene climate change, what the limits to gene flow might be, or
96 whether inbreeding and reduced gene pool are currently constraining its range of adaptation. We investigate the
97 genetic structure patterns at different spatial scales, as well as accounting for a demographic, time-related,
98 perspective by sampling young, adult and old trees of this species. We hypothesise low genetic diversity for the
99 studied *A. pinsapo* forests, according to their relict features, as compared to other non-relict
100 circum-Mediterranean firs. A second hypothesis is that *A. pinsapo* should show evidence of inbreeding and
101 recent bottlenecks due to long-term isolation of its remnant populations. Consequently, geographic location is
102 hypothesized here to affect genetic diversity, both within and among *A. pinsapo* populations. We seek to
103 provide information to guide management policies aiming to reduce future extinction risk for this endangered
104 tree.

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108 2. Materials and Methods

109 2.1. *Abies pinsapo* as experimental model

110 We focus on *Abies pinsapo* Boiss., a Tertiary relict tree species, endemic of the Baetic Range (Southern
111 Spain), closely related to North-Moroccan populations *A. marocana* Trab., and *A. tazaotana* Villar., (Terrab et
112 al., 2007; Jaramillo-Correa et al., 2010; Dering et al., 2014; Sanchez-Robles et al., 2014). Currently, *A. pinsapo*
113 is listed as endangered, as well as other circum-mediterranean firs, by the International Union for Conservation
114 of Nature (Arista et al., 2011). This species represents the southernmost European fir species (Sánchez-Salguero
115 et al., 2017). Likewise, as a climate relict, *A. pinsapo* displays limited possibilities of migration, while its
116 long-term persistence has been related to climatic refugia (Alba-Sánchez et al., 2010; Linares 2011;
117 Alba-Sánchez et al., 2019). Currently, *A. pinsapo* populations are mainly located on north-facing slopes
118 between 1000 and 1800 m.a.s.l. (Linares et al., 2009). Fragmented populations of *A. pinsapo* experienced an
119 expansion and densification from scattered remaining stands following the implementation of conservation
120 measures in the middle of the 20th Century (Linares et al., 2009; Lechuga et al., 2017). However, recent climate
121 change has been related to increasing drought and *A. pinsapo* forest decline (Linares et al., 2009, 2011; Lechuga
122 et al., 2017, 2019). Recurrent mortality events, mainly at low-elevation sites within the elevation range of *A.*
123 *pinsapo* have been noted, whilst the high-elevation sites yielded a recent growth enhancement and increasing
124 forest cover (Linares et al., 2009; Lechuga et al., 2019). Given the limited migration potential of this climate
125 relict, climate change may be imposing an enhanced threat for the conservation of this species (Hampe and Petit
126 2005; Kuparinen et al., 2010; Hampe and Jump 2011; Rehm et al., 2015).

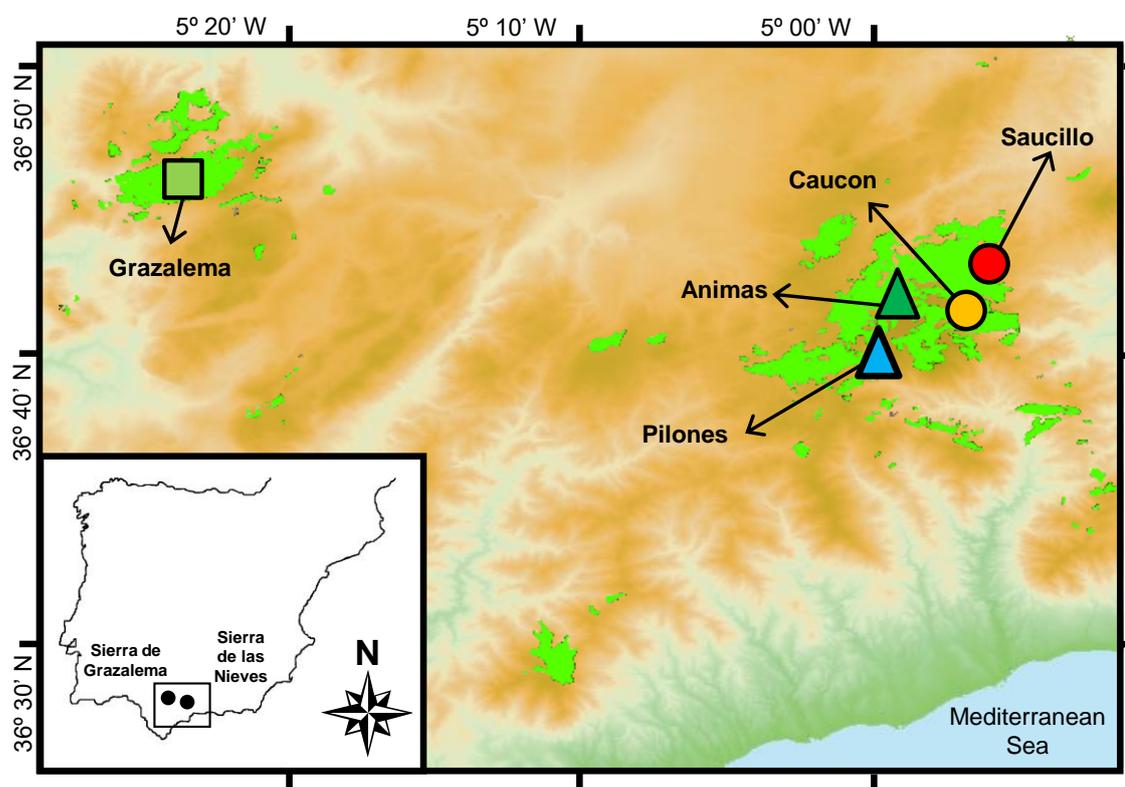
127 2.2. Sampling Design and Plant Material

128 *A. pinsapo* forests are mainly restricted to two locations in the Baetic Range, each subjected to a
129 conservation designation (Figure 1): the National Park Sierra de las Nieves (36°41'53"N, 4°59'50"W),
130 accounting for about 5800 ha, and the Biosphere Reserve Sierra de Grazalema (36°46'25"N, 5°24'35"W),
131 accounting for about 2000 ha. Although, scattered stands and isolated trees, assumed to represent the remains of
132 former larger populations, are rather common throughout the current *A. pinsapo* range (Figure 1). Sampling was
133 performed in these main ranges, by selecting individuals through several altitudinal gradients, between 1100
134 and 1700 m a.s.l., thereafter grouped as low-, middle- and high-elevation (Table 1), including some lower and
135 upper altitudinal ecotones (Linares et al., 2009). We also studied the genetic structure of trees belonging to
136 different cohorts by focusing on three watersheds (Saucillo, Caucon and Animas), located in Sierra de las

137 Nieves National Park, which represents the main area covered by dense *A. pinsapo* forests (Alba-Sánchez et al.,
138 2019).

139 Here, based on previous dendrochronological research (Linares et al., 2009, 2011), we sampled
140 individuals belonging to different ages in order to test whether the genetic structure of different cohorts is
141 demographically stable or might be undergoing genetic erosion (Wehenkel and Saenz-Romero 2012). We
142 sampled needles of old individuals selected at distance intervals of about 50 m, and their closest mature tree and
143 juvenile sapling. Mean ages were 138 ± 36 years in old trees, 65 ± 9 years in mature trees, and 26 ± 11 years in
144 juvenile trees, respectively. A total of 202 trees were sampled and the collected needles stored at -80°C prior to
145 DNA extraction.

146



147

148 **Figure 1.** Study area (left bottom inset), distribution of *Abies pinsapo* forests (green shape) and
149 sampling location (symbols) within the mountain ranges of the Sierra de las Nieves National Park
150 and the Sierra de Grazalema Biosphere Reserve. See also Table 1.

151

152

153 2.3. DNA Extraction, Microsatellite and Inter-Microsatellite Genotyping.

154 We studied three different genomic microsatellites (simple sequence repeats; SSR) as neutral molecular
 155 markers with different inheritances: nuclear markers (nuclear microsatellites, nSSR, and inter-microsatellites,
 156 ISSR), which are biparentally inherited; and chloroplast markers (chloroplast microsatellites, cpSSR),
 157 paternally inherited in conifers (Liepelt et al., 2002; Petit et al., 2005; Neale and Wheeler 2019). Total genomic
 158 DNA was extracted and purified according to QIAGEN DNeasy plant mini kit protocol (Pérez-González et al.,
 159 2018). We used 8 nuclear microsatellites to perform genomic DNA amplification: NFF2, NFF3, NFH15, NFH3
 160 and NFF7 developed for *A. nordmanniana* Stev. (Hansen et al., 2005); and Pin8, Pin20 and Pin48 developed for
 161 *A. pinsapo* (Sánchez-Robles et al., 2012). In addition, we amplified 3 chloroplast microsatellites: Pt30204,
 162 Pt71936 and Pt15169 developed for *Pinus thunbergii* (Vendramin et al., 1996). Microsatellite selection was
 163 done based on previous studies that prove that they yield enough polymorphic bands in other species
 164 phylogenetically related with *A. pinsapo*. 19 ISSR primers from the University of British Columbia, Canada
 165 (UBC) were tested in two individuals to select those that yield more polymorphic bands (see the detailed
 166 methodology in the Electronic Supplementary Material, Appendix 1).

167

168 **Table 1.** Main characteristics and sample size of the studied *Abies pinsapo* populations. The number
 169 of young (Y), Adult (A), and old (O) trees is indicated between parenthesis.

Population	Site (Code)	Elevation classes	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)	N (age classes)
Saucillo	S	Low (SL)			1178-1259	16 (Y=6, A=6, O=4)
		Middle (SM)	36° 42' 43" - 36° 43' 33"	4° 57' 55"- 4° 59' 16"	1295-1340	16 (Y=5, A=6, O=5)
		High (SH)			1450-1521	14 (Y=5, A=4, O=5)
Caucon	C	Low (CL)			1112-1190	21 (Y=7, A=7, O=7)
		Middle (CM)	36° 42' 15" - 36° 42' 45"	4° 57' 50" - 4° 58' 28"	1225-1289	39 (Y=13, A=13, O=13)
		High (CH)			1307-1399	15 (Y=5, A=5, O=5)
Animas	A		36° 41' 46" - 36° 41' 56"	5° 00' 59" - 5° 01' 04"	1589-1684	50 (Y=20, A=10, O=20)
Grazalema	G	Low (GL)			1165-1287	6
		Middle (GM)	36° 45' 53" - 36° 46' 28"	5° 24' 8" - 5° 25' 39"	1305-1379	6
		High (GH)			1391-1479	6
Pilones	P		36° 41' 36"	5° 01' 09"	1716-1740	13

170

171 2.4. Statistical Analysis

172 2.4.1. Hardy-Weinberg equilibrium and null alleles

173 Neutrality among the molecular markers was tested by scanning the nSSR dataset for loci under
174 differential selection, using outlier loci analysis in BayeScan 2.01 (Foll and Gaggiotti 2008). We examined the
175 presence and frequency of null alleles using the Expectation Maximization (EM) algorithm in FreeNA (Chapuis
176 and Estoup 2007). Null alleles frequencies were estimated for each locus, as well as for the mean frequency of
177 null alleles in each population. Since the presence of null alleles may overestimate the population genetic
178 differentiation, an F_{st} statistic was computed excluding null alleles (ENA) and without the ENA correction
179 method. Simulation studies suggest that null alleles with frequencies between 5% and 8% should have only
180 minor effects on estimates of population differentiation (Chapuis and Estoup 2007). We used bootstrapping to
181 estimate 95% confidence intervals, running 50000 replicates per locus.

182 We calculated allele frequencies for each polymorphic locus obtained by ISSR assuming Hardy-Weinberg
183 equilibrium. GeneAIExv6.502 was used to estimate the frequency of the null alleles (q) by taking the square
184 root of the frequency of the null homozygotes (absence of a band). Then, we obtained the frequency of the
185 dominant allele as $p=1-q$. We removed bands with frequencies higher than $1-(3/N)$, where N is the population
186 sample size, to avoid ISSR underestimates of genetic variation (Chapuis and Estoup 2007).

187 2.4.2. Within-populations and within-cohorts genetic diversity

188 Since the nuclear (nSSR and ISSR) and chloroplast (cpSSR) neutral molecular markers used in this study
189 have different characteristics (diploid codominant, diploid dominant and haploid, respectively), we estimated
190 different parameters with each one to describe the neutral genetic diversity of the species, within different
191 populations and within different cohorts. For all neutral markers, we calculated the following parameters:
192 Percentage of polymorphic loci (PPL), number of private alleles (NPA) and number of effective alleles (N_e).
193 For nSSR and ISSR, expected heterozygosity (H_e) was also estimated. For nSSR we calculated observed
194 heterozygosity (H_o); and Wright's fixation indices for within-subpopulation to test the inbreeding index (FIS)
195 (Weir and Cockerham 1984). We calculated unbiased diversity (h) and haplotype frequencies based on cpSSR
196 markers. All these analyses were carried out with GeneAIEx 6.501 (Peakall and Smouse 2006, 2012).

197 Finally, rarefied allelic richness (A_r) was estimated based on nSSR molecular markers with FSTAT, as
198 well as number of migrants (N_m) among populations and spatial and temporal cohorts. In addition, a Student t
199 test was implemented with statistical significance at the 5% nominal level of the difference between the mean
200 value of H_o and H_e across all samples for all nSSR loci in order to test again a possible effect of selection. The

201 statistically significance of the differences of these parameters among spatial and temporal cohorts were
202 estimated by means of an unpaired Student t test for unequal variances.

203 To detect any recent severe reduction in effective population size or possible expansion events in *A.*
204 *pinsapo* populations, BOTTLENECK 1.2.02 was used on the nSSR dataset (Cornuet and Luikart 1996; Luikart
205 et al., 1998; Piry et al., 1999; Petit et al., 2005). Bottlenecks cause low-frequency alleles to become transiently
206 less abundant (<0.1), while more intermediate-frequency alleles increase (Luikart et al., 1998). BOTTLENECK
207 correlates expected heterozygosity (H_e) with observed heterozygosity (H_o) at mutation-drift equilibrium. The
208 two-phased model (TPM) of mutation was applied as the most appropriate for microsatellite data (Piry et al.,
209 1999). For TPM, we used 5% and 15% of multistep changes (Probability 95% and 85 %, respectively) and a
210 variance among multiple (12) steps (Piry et al., 1999). For each population, 2000 simulations were performed in
211 all datasets. Significance was assessed using the implemented Wilcoxon sign-rank test, which determines
212 whether or not the average of standardized differences between H_o and H_e is significantly different from zero
213 (Cornuet and Luikart 1996). Significant heterozygote excess relative to the number of alleles indicates a recent
214 population bottleneck.

215

216 2.4.3. Among-populations and among-cohorts genetic differentiation

217 We summarized genetic differentiation among the different populations of *A. pinsapo* using pairwise F_{st}
218 and pairwise Nei's standard genetic distances on all neutral markers. Spatial limitation of gene flow resulting in
219 an isolation by distance pattern was analysed by a Mantel-Test, performed with GeneAIEx version 6.501, using
220 genetic and geographical distances (Peakall and Smouse 2006; Peakall and Smouse 2012). Significance was
221 estimated via 9999 permutations. We estimated partitioning of genetic variation among locations, elevations
222 and age cohorts, as well as within populations, using a hierarchical analysis of molecular variance (AMOVA) in
223 GenAlex (Peakall and Smouse 2006; 2012). For nSSR markers, the analysis was based on allele identity and
224 allele size by using F_{st} and R_{st} respectively. For ISSR and cpSSR markers, the analysis used PhiPT, a measure
225 that allows the suppression of intra-individual variation, comparing dominant and haploid markers (Peakall and
226 Smouse 2006). 9999 random permutations were carried out for significance testing in all cases. To establish the
227 effects of geographic, cohort and altitudinal distances on genetic differentiation, we applied a multivariate
228 principal coordinates analysis (PCoA) based on pairwise Nei's standard genetic distances among populations in
229 GenAIEx (Peakall and Smouse 2006).

230 To estimate the optimum number of subpopulations (K), we applied a model-based clustering algorithm in
231 a Bayesian framework and the Markov chain Monte Carlo (MCMC) algorithm with STRUCTURE (ST,

232 thereafter; Earl and vonHoldt 2012) under the assumption that each cluster is in optimal H-W equilibrium and
233 linkage equilibrium (LE). ST analysis used correlated allele frequencies and the admixture model, which
234 allowed for mixed recent ancestries of individuals and assigned the proportion of the genome of each individual
235 to the inferred clusters without prior population information. We ran the analysis for K values of 1-10 with 10
236 independent runs each and a burn-in period of 100000 and thereafter 200000 MCMC, without the inclusion of
237 geographic coordinates. The number of genetically homogeneous clusters (K) was identified by following the
238 method developed by Evanno et al. (2005). The results were summarized in ST HARVESTER (Earl and
239 vonHoldt 2012). ST analysis calculated the membership coefficient of individuals (individuals Q-matrix) for
240 each of the defined genetic clusters and the proportion of ancestry of each population in each cluster (population
241 Q-matrix) by averaging the membership coefficient of all individuals in a population. The populations were
242 assigned to a specific cluster based on an arbitrary threshold of $Q > 0.80$ regardless of the values of the rest of
243 the clusters.

244

245 2.5. Demographic history

246 The software DIYABC v2.1.0 was used to infer past demography of the studied populations (Cornuet et
247 al., 2014). Both nuclear (nSSR) and chloroplast (cpSSR) neutral markers were used to perform the analysis
248 (Supplementary material, Appendix 2). The same prior parameters were defined for all scenarios. The default
249 values of the priors were used for all parameters. Minimum estimate of generation time of 20 years was used in
250 the calculation of number of generations, since *A. pinsapo* trees start to produce seeds at this age (Authors'
251 personal observation; Arista and Talavera 1994a). Summary statistics obtained by run one million simulations
252 included mean number of alleles, mean genetic diversity, mean size variance across loci and mean
253 Garza-Williamson's M index across loci for each population and for population pairs, Fst, classification index,
254 shared allele distance and du2 distance were also included. The 10% simulated data sets closest to observed data
255 set was used to estimate posterior distributions of parameters through a local linear regression procedure. Seven
256 evolutionary scenarios were tested with DIYABC 2.1.0 based on the results obtained here and in previous
257 studies (Dering et al., 2014; Sanchez-Robles et al., 2014) to test genetic differentiation among populations and
258 assuming hypothetical divergence times (Electronic Supplementary Material, Appendix 2). Models were
259 compared by estimating their posterior probabilities using the direct estimation and logistic regression methods
260 (Cornuet et al., 2014).

261

262

263 3. Results

264 3.1. Hardy-Weinberg equilibrium (HWE) and null alleles

265 The W parameter estimated for nSSR markers did not show any locus under differential selection
266 according to any of the applied criteria (all samples together, each population separately and different cohorts,
267 both temporal and spatial). In addition, mean observed heterozygosity ($H_o = 0.528 \pm 0.031$) was not significantly
268 different from the mean expected heterozygosity under HWE ($H_e = 0.596 \pm 0.034$; Student t test, $P = 0.095$; Table
269 2). Likewise, FreeNA analysis did not show significant evidence for null alleles, since the estimated null allele
270 frequency using the EM algorithm was 5.1 %; variation in F_{st} estimation was negligible after excluding null
271 alleles (ENA $F_{st} = 0.0602$), compared to F_{st} without ENA correction ($F_{st} = 0.0658$). EM algorithm did not show
272 evidence of high frequencies of null alleles for any of the nSSR loci (Electronic Supplementary Material, Table
273 S1). For ISSR markers, 7 out of the 19 tested oligos generated polymorphic and reproducible bands: 807,
274 807b18up, 825, 835b10lw, 855, otrob4lw and otrob16lw (University of British Columbia, Canada), which were
275 used to carry out further analysis.

276

277 3.2. Genetic diversity of *A. pinsapo* in Iberian Peninsula

278 The studied neutral molecular markers yielded a high percentage of polymorphic loci (PPL) (nSSR,
279 $PPL = 95.83\%$ and cpSSR, $PPL = 83.33\%$) with the exception of ISSR which yielded only a 14.81%, indicating
280 that are less effective markers to test the genetic diversity of this species. Neutral genetic diversity of *A. pinsapo*
281 in the Iberian Peninsula was moderately high using all analyzed molecular markers with the exception of ISSR.
282 Thus, only polymorphic loci were included in the subsequent analyses. Particularly, for nSSR, mean rarefied
283 allelic richness (A_r) reached a value of 2.78 for a standardized sample size of $n = 6$ gene copies (Table 2). The
284 overall mean inbreeding coefficient ($F_{is} = 0.150$) was statistically different from zero ($P < 0.001$; Table 2).
285 Effective number of alleles (N_e) was 2.825 ± 0.195 , H_o was 0.528 and H_e , 0.596. For cpSSR markers, diversity
286 (h) showed a value of 0.523 ± 0.077 and N_e was 2.541 ± 0.306 . Finally, for ISSR markers, H_e was 0.035 ± 0.005 ,
287 but rose to a value of 0.167 ± 0.02 based on polymorphic loci and N_e was 1.058 ± 0.009 , reaching the lowest
288 values of all analysed genetic diversity parameters, as expected considering their particularly low PPL (Table
289 2).

290

291

292 **Table 2.** Genetic diversity within population parameters of *A. pinsapo* individuals sorted by population,
 293 elevation and age cohorts (See abbreviations in Table 1) based on the three studied neutral molecular markers
 294 (nSSR, cpSSR, ISSR). N, population size; NPA, number of private alleles; Ne, number of effective alleles; Ar,
 295 rarified allelic richness; Ho, observed heterozygosity; He, expected heterozygosity; h, genetic diversity; Fis,
 296 inbreeding index.

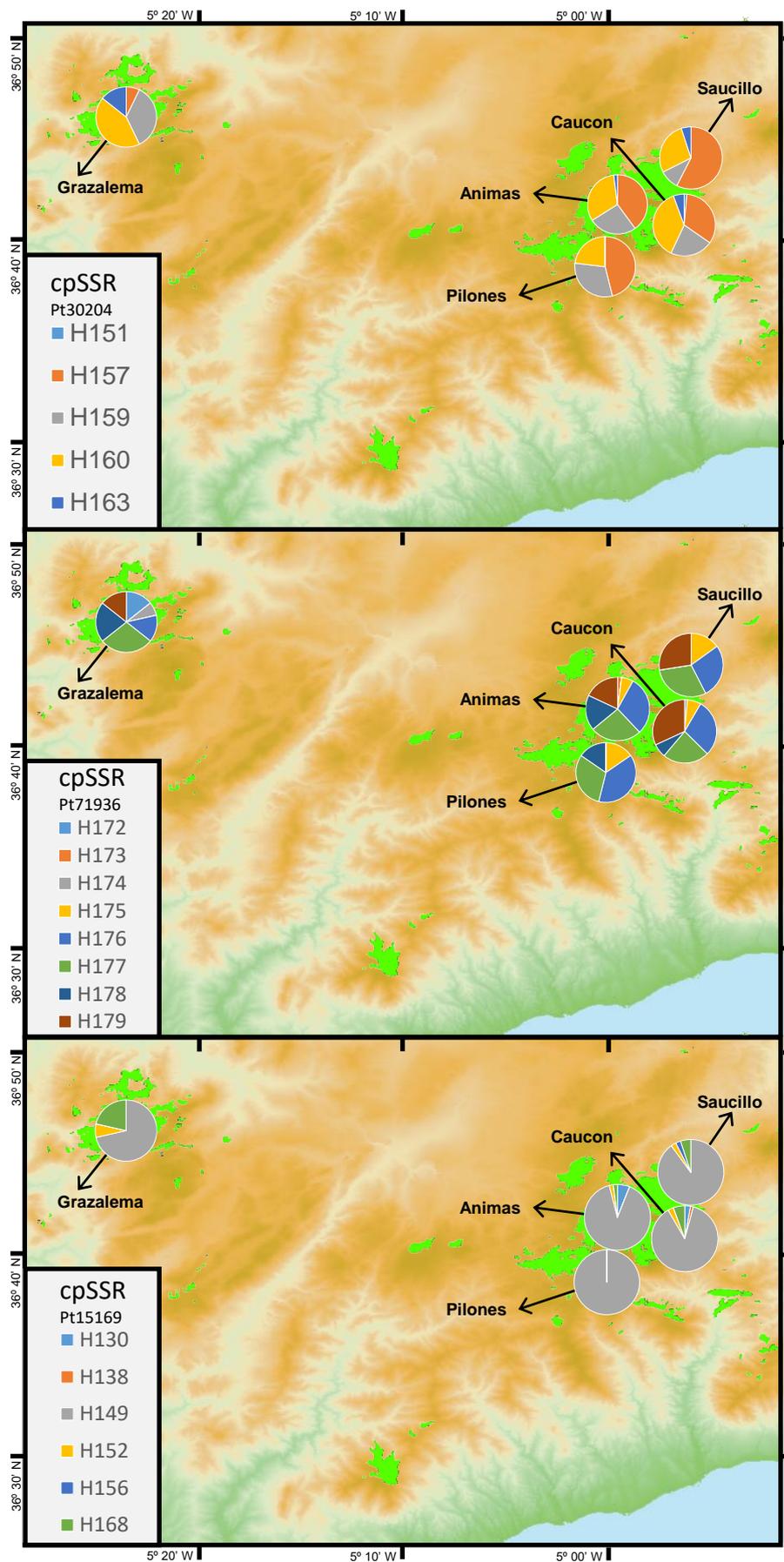
Code	nSSR			cpSSR			ISSR					
	NPA	Ne	Ar (gene copies)	Ho	He	Fis (p-value)	NPA	Ne	h	NPA	Ne	He
S	1.13	3.032	2.872 (6)	0.431	0.589	0.265 (<0.001)	0.333	2.469	0.514	0	1.045	0.029
SL		2.966	3.890 (12)	0.471	0.603	0.225 (<0.001)		2.15	0.431		1.037	0.024
SM		2.707	3.495 (12)	0.403	0.549	0.264 (<0.001)		2.421	0.575		1.051	0.03
SH		2.797	3.886 (12)	0.402	0.581	0.311 (<0.001)		2.094	0.524		1.037	0.024
SY		2.585	2.698 (6)	0.369	0.558	0.336 (<0.001)		2.459	0.545		1.043	0.026
SA		2.954	2.878 (6)	0.438	0.568	0.238 (<0.001)		2.116	0.53		1.044	0.026
SO		3.227	3.082 (6)	0.521	0.636	0.188 (<0.001)		2.069	0.449		1.041	0.026
C	0.38	3.126	2.896 (6)	0.564	0.613	0.08 (0.04)	0.667	2.847	0.565	1	1.079	0.047
CL		2.951	4.791 (26)	0.581	0.617	0.060 (0.157)		2.317	0.465		1.072	0.041
CM		3.109	4.946 (26)	0.568	0.614	0.077 (0.03)		3.223	0.63		1.082	0.049
CH		2.581	4 (26)	0.5	0.572	0.131 (0.04)		2.305	0.474		1.067	0.039
CY		2.981	5.375 (46)	0.527	0.598	0.121 (0.014)		2.575	0.511		1.064	0.04
CA		3.165	5.463 (46)	0.547	0.618	0.118 (0.009)		2.508	0.507		1.075	0.043
CO		3.06	5.75 (46)	0.603	0.625	0.036 (0.271)		3.022	0.636		1.084	0.049
A	0.63	3.11	2.830 (6)	0.594	0.612	0.028 (0.236)	0.333	2.89	0.554	0	1.082	0.049
AY		3.019	4.148 (18)	0.644	0.627	0.028 (0.714)		2.737	0.561		1.087	0.051
AA		2.845	4.146 (18)	0.593	0.633	0.053 (0.297)		2.606	0.496		1.043	0.025
AO		2.969	3.748 (18)	0.544	0.574	0.054 (0.202)		2.812	0.592		1.079	0.045
G	0	2.563	2.590 (6)	0.598	0.546	-0.100 (0.913)	0.333	3.303	0.685	0	1.812	0.048
GL		2.283	2.5 (4)	0.688	0.604	-0.222 (0.999)		2	1		1.069	0.037
GM		2.288	2.12 (4)	0.604	0.504	-0.224 (0.978)		3.257	0.822		1.055	0.031
GH		2.586	2.303 (4)	0.563	0.6	0.069 (0.385)		1.933	0.444		1.073	0.04
P	0	3.035	2.750 (6)	0.481	0.631	0.245 (<0.001)	0	2.406	0.487	0	1.061	0.037
Total		2.825	2.788 (6)	0.528	0.596	0.120 (<0.001)		3.722	0.523		1.058	0.035

297 3.3. Spatial genetic structure and demographic history

298 Overall, the data showed no significant differences for among population genetic differentiation. Hence,
 299 nSSR markers showed Ar for a standardized sample size of 6 ranging from 2.590 in Grazalema to 2.896 in
 300 Caucon (Table 2). He values ranged from 0.546 in Grazalema to 0.631 in Pílon. On the other hand, FIS was
 301 very high and statistically significant in Saucillo and Pílon populations (FIS=0.265, P<0.001 and FIS=0.245,

302 $p < 0.001$ respectively) and moderate but statistically significant in Caucon ($FIS = 0.080$, $p = 0.004$). However,
303 Animas and Grazalema populations showed a very low value that was not significantly different from 0 ($FIS =$
304 0.028 , $P = 0.236$ and $FIS = -0.100$, $P = 0.913$, respectively). For cpSSR markers, h ranged from 0.487-0.685. For
305 ISSR markers, He values were between 0.029-0.049 (Table 2). In addition, the Grazalema population showed
306 the widest variety of haplotypes and the most equally distributed (Figure 2). Student's t test for unequal
307 variances showed no statistically significant differences, nor between Sierra de las Nieves and Grazalema for
308 the analysed neutral markers, neither among the different studied populations ($p > 0.05$ in all cases), with the
309 exception of FIS between Grazalema and Sierra de las Nieves ($p = 0.001$), as well as among Sierra de las Nieves
310 populations ($P < 0.05$), indicating that genetic diversity is very similar in all populations. Regarding genetic
311 diversity among populations, low to moderate differences were observed based on all three neutral markers
312 (Supplementary material, Tables S2-S4).

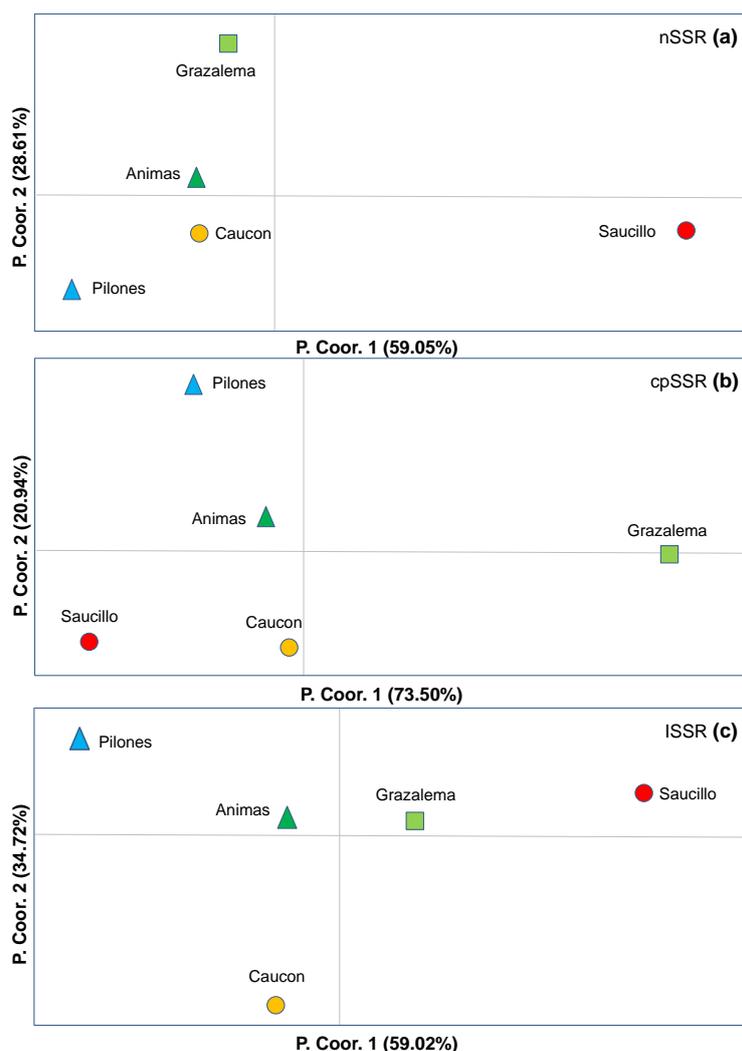
313 Hierarchical AMOVA for nSSR markers based on F_{st} and R_{st} showed a 7% and 9% of the total genetic
314 variance due to differences among populations, a 10% and 6% among individuals within populations and 83 %
315 and 85% within individuals, respectively. For cpSSR and ISSR, hierarchical AMOVA based on Φ_{PT} showed
316 a 5% and 12% of the total genetic variance due to differences among populations and a 95% and 88% due to
317 differences within populations, respectively. The genetic differences among populations were statistically
318 significant in all cases (nSSR: F_{st} , $p = 0.001$ and R_{st} , $p = 0.001$; cpSSR: Φ_{PT} , $p = 0.04$; ISSR, Φ_{PT} , $p = 0.001$).
319 Moreover, pairwise F_{st} and Nei distances were statistically significant based on all analysed markers and
320 showed the highest differences in Grazalema, based on chloroplast markers (cpSSR) and Saucillo, based on
321 nuclear markers (nSSR and ISSR), congruently with the geographical distribution of populations, as Saucillo
322 represents the westernmost population and Grazalema the easternmost one (Figures 1 and 2). For nSSR,
323 pairwise F_{st} and Nei were congruent in their results, and all pairwise F_{st} differences were significantly larger
324 than 0 ($p < 0.05$). For cpSSR, Nei distances ranged from 0.224 to 0.107 and for ISSR, from 0.0249 to 0.00597.
325 PCoA results (Figure 3; Electronic Supplementary material, Figure S1) were consistent with the previous
326 analysis, showing differentiation in Saucillo, based on nuclear markers (nSSR and ISSR), and Grazalema, based
327 on chloroplast markers (cpSSR; Figures 2 and 3).



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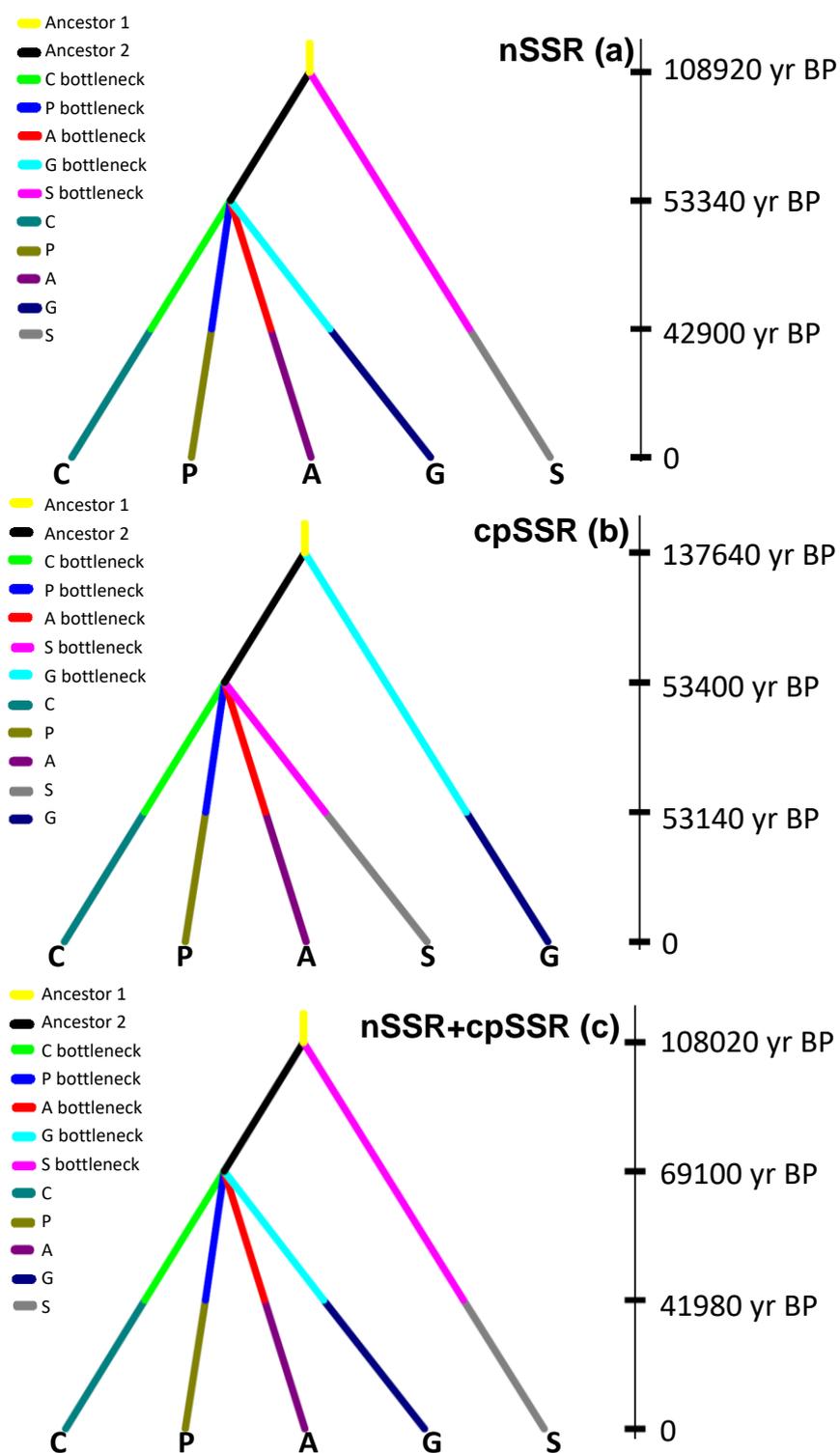
Figure 2. Distribution pattern of Pt30204, Pt71936, and Pt15169 cpSSR haplotypes.



330

331 **Figure 3.** PCoA analyses based on pairwise Nei's standard genetic distances sorted by populations:
 332 nSSR (a), cpSSR (b), and ISSR (c).

333 STRUCTURE analyses (Electronic Supplementary material, Figure S2) separated some of the *A. pinsapo*
 334 populations based on nuclear markers (nSSR, ISSR). Based on nSSR, Saucillo was separated, in agreement
 335 with F_{st} and Nei distances (Table 2) and PCoA results (Figure 3). ISSR markers also yielded a separation
 336 among the different populations. However, STRUCTURE analyses based on cpSSR did not separate the
 337 different studied populations. Isolation by distance (IBD) assessed over all populations did not shown
 338 statistically significant result based on nuclear markers (Mantel test, nSSR, $p=0.412$; ISSR, $p=0.284$). However,
 339 it was weak but significant, based on chloroplast markers (cpSSR, $p=0.044$) yet with the RMA regression
 340 explaining only 1% of the variance in the whole distribution area ($R^2=0.0105$). The BOTTLENECK analysis
 341 based on nSSR showed evidence of significant heterozygote excess (recent decline) in Saucillo ($p = 0.02$),
 342 Caucon ($p = 0.037$), Pilonos ($p = 0.019$) and Grazalema populations ($p = 0.009$).



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Figure 4. Likelihood scenarios for differentiation among the studied *Abies pinsapo* populations based on nSSR markers (a); cpSSR markers (b); and using both, nSSR and cpSSR markers (c); t(i) indicates time scale measured in generations; the segments indicates the effective population sizes prior and after simulated bottlenecks. S, Saucillo; C, Caucon; A, Animas; P, Pilonas; and G, Grazalema. See also geographic locations in Figure 1.

349 DIYABC 2.1.0 results (Figure 4) showed the following evolutionary scenarios: (Figure 4a) based on
350 nuclear markers, Saucillo population diverged from an ancient ancestral population and then, the rest of
351 populations split at the same time with a recent bottleneck in all populations. (Figure 4b) based on chloroplast
352 markers, Grazalema population diverged from an ancient ancestral population and then, the rest of population
353 split at the same time with also a recent bottleneck in all populations. These results were consistent with the
354 previously showed above, which pointed to Grazalema as the most different population based on chloroplast
355 markers and Saucillo based on nuclear markers, and also with the BOTTLENECK results, which pointed to the
356 existence of recent bottlenecks in all populations with the exception of Animas. However, Animas also showed
357 evidence of a recent bottleneck based on DIYABC results. The posterior probabilities of these scenarios were
358 0.709 (95% CI = 0.3112–1.0000) and 0.899 (95% CI = 0.8414–0.9532) for direct estimation and logistic
359 regression respectively, based on nSSR; and 0.1102 (95% CI = 0.0000–0.3846) and 0.6956 (95% CI = 0.6000–
360 0.8459) based on cpSSR.

361 Finally, (Figure 4c) using nSSR and cpSSR together to carry out the analysis, the most probable scenario
362 was Saucillo divergence from an ancient population in the first place followed by the rest of populations
363 splitting at the same time, with a recent bottleneck in all of them (Figure 4c). The posterior probabilities of this
364 scenario were 0.415 (95% CI = 0.00325-0.84592) and 0.516 (95% CI = 0.3193-0.7130). The effective
365 population size based on nSSR was lower in Saucillo and Caucon and similar among the other populations.
366 However, cpSSR predicted the highest effective population size for Animas (Electronic Supplementary
367 material, Tables S5 and S6).

368 *3.4. Genetic differentiation among spatial and temporal cohorts*

369 Individuals belonging to high, middle and low elevation, as well as young, adult and old trees, showed
370 non-statistically significant differences for the studied molecular markers ($P > 0.05$ in all cases; Student's t
371 tests), indicating that there are no differences in terms of neutral genetic diversity related to altitudinal gradients
372 or age-related cohorts. In addition, the number of migrants (N_m) based on the F_{st} of nSSR from different
373 elevations showed high values in Saucillo, Caucon and Grazalema, ranging from 3.32 to 20.5. Hierarchical
374 AMOVA analysis did not showed any significant differences related to elevation and cohorts, based on
375 chloroplast markers but some significant differences were found based on nuclear markers: Saucillo ($F_{st}=0.02$,
376 $p=0.02$) and Caucon ($F_{st}=0.01$, $p=0.04$) by elevation based on nSSR ($F_{st}=0.091$, $p=0.005$), and Grazalema by
377 elevation ($\Phi_{PT}=0.16$, $p=0.003$) together with Saucillo and Animas by age ($\Phi_{PT}= 0.22$, $p=0.001$ and 0.16 ,
378 $p=0.002$, respectively) based on ISSR.

379 4. Discussion

380 4.1. Genetic diversity of neutral molecular markers.

381 We hypothesised a low genetic diversity for *A. pinsapo*, according to the long-term isolation and relict
382 character of this Mediterranean fir (Hampe and Petit 2005; Linares, 2011; Hampe and Jump 2011). However,
383 the patterns obtained here were congruent among markers, supporting a relatively high within-population
384 genetic diversity. Hence, nSSR markers revealed that *A. pinsapo* holds a relatively high genetic diversity ($He =$
385 0.596 ± 0.034) and allelic richness ($Ar = 2.79$). High molecular diversity has been previously reported in this
386 (Dering et al., 2014; Sanchez-Robles et al., 2014) and other relict conifers, such as Serbian spruce (*Picea*
387 *omorika* (Panč.) Purk.; Aleksić and Geburek 2013) and Chihuahua spruce (*Picea chihuahuana* Martinez; Ledig
388 et al., 1997; Jaramillo-Correa et al. 2006; Wehenkel and Saenz-Romero 2012). These findings suggest that the
389 observed high levels of genetic diversity of several relict conifers rely on frequent past admixture events of
390 genetically differentiated populations (Alba-Sánchez et al., 2010; Eliades et al., 2011; Linares 2011; Aleksić
391 and Geburek 2013), supporting a different way of retention of genetic variants, compared to other conifers with
392 broad ranges, whose molecular diversity seems to be maintained by large effective populations sizes
393 (Vendramin et al., 1999; Parducci et al., 2001; Liepelt et al., 2010).

394 Secondly, we also hypothesised the existence of inbreeding and recent bottlenecks, driven by long-term
395 isolation of the remnant *A. pinsapo* populations. The inbreeding index obtained here ($Fis = 0.150$) was higher
396 than those obtained previously in *A. pinsapo* (Dering et al., 2014) and other relict circum-mediterranean fir,
397 such as *A. cilicica* (Awad et al., 2014). However, the high inbreeding index showed by some populations
398 (specifically, Caucon, Saucillo and Pilonés), may be related to our sample design since Caucon and Saucillo
399 populations (Figure 1) were sampled by selecting old individuals and their closest mature trees and saplings. As
400 a consequence, neighbouring trees might be related, likely affecting the *Fis* estimates. Indeed, Grazalema and
401 Animas populations (Figure 1), which were randomly sampled, did not showed significant inbreeding.
402 Nonetheless, the inbreeding of these *A. pinsapo* populations might be also related to land-use legacies (Reed
403 and Frankham 2003; Kremer et al., 2012) since Caucon and Saucillo populations were subjected over centuries
404 to intensive human perturbations, such logging and grazing, until these forests were declared as protected areas
405 (Linares et al., 2009; Lechuga et al., 2017). Although Grazalema and Animas populations were also subjected to
406 logging and grazing, the first belonged to private owners, while the second belonged to a municipality where
407 logging and grazing were less intense (Linares et al., 2009). Finally, Pilonés population represents one of the
408 current treeline ecotones of *A. pinsapo*, which seems to be expanding upward as a consequence of both global

409 warming and land-use changes (Lechuga et al., 2019). Thus, the significant inbreeding index obtained in these
410 randomly sampled individuals could be related to their recent expanding dynamics from a limited number of
411 leading-edge individuals (Hampe and Petit 2005), although the effects of a limited sample size ($n=13$) and the
412 high frequency of null alleles (0.101; Table S1) may also play a role here.

413 4.2. Spatial genetic structure and demographic history.

414 Spatial differentiation regarding the among-populations genetic structure should be expected in relict tree
415 species (Clark et al., 2000; Eliades et al., 2011; Hampe and Jump 2011). Accordingly, geographic location was
416 hypothesized here to affect genetic diversity, both within and among *A. pinsapo* populations. The studied *A.*
417 *pinsapo* populations revealed an overall low genetic differentiation and low spatial genetic structure. Despite
418 the low spatial genetic structure inferred here, the genetic differentiation was statistically significant. Besides,
419 the inferred demographic history supports a bottleneck effect experienced in all the studied populations. Indeed,
420 most circum-mediterranean relict species may have suffered a genetic bottleneck at some point in their
421 evolutionary history, resulting in a dramatic decrease in genetic diversity (Fady-Welteren, 2005; Fady and
422 Conord 2010; Linares, 2011).

423 The earlier differentiation among the studied populations occupies the time between ca. 140 and ca. 100
424 thousand years before present (kyr BP). This period is of particular interest with regard to orbital parameters,
425 contrasted vegetation changes and climatic conditions (Cheddadi et al., 1998). Although estimates of time
426 frames affecting the studied *A. pinsapo* populations must be taken with caution, this former differentiation
427 among the studied populations of *A. pinsapo* might be related to the major cooling episode that occurred after
428 the Last Interglacial (Combourieu-Nebout et al., 2002; Fletcher and Sanchez Goñi 2008). Further bottlenecks,
429 inferred from ~70–40 kyr BP in all the studied populations, may be related to abrupt climate changes, such as
430 the Dansgaard-Oeschger (DO) and the Heinrich (H) events, occurring during the last glacial cycle and the
431 Holocene (Heinrich, 1988; Dansgaard et al., 1993). The periods between ~115–100 kyr BP and ~75–45 kyr BP
432 have been related to regional-scale dry conditions in the west Mediterranean, based upon low groundwater
433 carbonate deposition and pollen-based palaeoclimate reconstructions (Cheddadi et al., 2005; Camuera et al.,
434 2019). Then, it is assumed that steppe-like vegetation predominated during these cold-dry events of the last
435 glacial stage such that the estimated *A. pinsapo* genetic bottlenecks might correspond to dramatic decreases in
436 genetic diversity linked to DO oscillations and H events.

437 The Quaternary genetic and demographic changes of other relict conifer, such as the cold-adapted spruce
438 *P. omorika*, suggest that scattered populations were subjected to long-term genetic isolation and related genetic

439 drift effects, that continuously increased among-populations genetic distinctiveness during the last glacial and
440 post-glacial (Aleksić and Geburek 2013). Nonetheless, due to their proximity to coastal glacial refugia,
441 populations of *A. pinsapo* have likely experienced buffered climatic fluctuations during the last glacial
442 termination (about 17.7–11.5 kyr BP; Linares, 2011). Indeed, while the glaciers were receding, periods of
443 intense cold and dry climate have been recorded, such as the Younger Dryas (12.9-11.7 kyr BP), which was not
444 inferred in any of our bottleneck modelling. In summary, the results obtained here, as well as those previously
445 published (Dering et al., 2014), support that *A. pinsapo* has been sensitive to climatic changes occurring during
446 the last glacial, as DO oscillations and H events, whilst the local-scale climate gradients might be ensured the
447 persistence of remnant stands (Linares, 2011). The relatively stable Holocene climate has also experienced
448 some intervals of rapid climate change that might have affected some *A. pinsapo* populations (Alba-Sánchez et
449 al., 2010; Dering et al., 2014). Additionally, the increasing human-induced habitat fragmentation after the last
450 glaciation likely limited the recolonization chance of remaining *A. pinsapo* populations (Alba-Sánchez et al.,
451 2019). Long-term logging and overgrazing activities have been related to declining genetic variation, as a result
452 of severe habitat fragmentation and temporal fluctuations in demographic parameters (Clark et al., 2000;
453 Wehenkel and Saenz-Romero 2012). Here, using paternally inherited cpSSR markers, we obtained the highest
454 effective population size for Animas (Table S4), according to the currently better-preserved old-growth *A.*
455 *pinsapo* forest (Alba-Sánchez et al., 2019).

456 The presence of two main *A. pinsapo* forests in South Spain (Figure 1), surrounded by several scattered
457 individuals and isolated small stands, suggests a wider former distribution (Linares 2011; Dering et al., 2014).
458 Hence, the weak geographic differentiation patterns obtained here might be explained by ensuing
459 pollen-mediated gene flow, as few migrants per generation are required to prevent divergence between
460 subpopulations for neutral markers (Clark et al., 2000; Petit et al., 2005; Chapuis and Estoup 2007; Kremer et
461 al., 2012). However, the restricted pollen dispersal of *A. pinsapo*, estimated as less than 3 km (Arista and
462 Talavera 1994b; Alba-Sánchez et al., 2010), and the low value of among-populations N_m obtained (1.69)
463 contrast with this hypothesis. The higher genetic differentiation between the populations of Grazalema and
464 Sierra de las Nieves based on chloroplast markers is consistent with their geographical distribution and agrees
465 with some relationships previously obtained between genetic and geographic distance also using cpSSR data
466 (Terrab et al., 2007). Hence, our results support that limited gene flow by pollen and almost complete lack of
467 seed flow may prevent genetic connectivity and enhance genetic differentiation among populations distant by a
468 few kilometres (Kremer et al., 2012; Aleksić and Geburek 2013).

469 We found that Grazalema contains a higher number of haplotypes for all three chloroplast markers,
470 compared to Sierra de las Nieves populations (Figure 2), suggesting the legacy of contrasting history, while
471 nuclear markers did not support this differentiation. This decoupled population genetic structure, based on
472 different-inherited DNA markers has been related to increasing genetic differentiation among different
473 ancestral populations (Clark et al., 2000; Petit et al., 2005; Jaramillo-Correa et al., 2006). Thus, comparison
474 between the genetic diversity of maternally inherited mitochondrial and paternally inherited chloroplast DNA
475 markers in the relict spruce *P. chihuahuana* showed higher cpDNA diversity, while these cpDNA markers
476 showed low population differentiation (Jaramillo-Correa et al., 2006). Our PCoA analyses based on Nei's
477 pairwise genetic distances revealed the highest differences in Grazalema, based on chloroplast markers (cpSSR)
478 and Saucillo, based on nuclear markers (both, nSSR and ISSR), which represents the westernmost and
479 easternmost populations, respectively (Figure 1).

480 Although these chloroplast and nuclear diversity estimates do not show genetic differentiation for
481 quantitative traits of adaptive relevance, this differentiation between the westernmost and easternmost
482 populations might be related to local climate gradients (Linares et al., 2011). Most of the annual rainfall, carried
483 by low pressure systems coming from Atlantic depressions, falls on the western part of the study area and
484 decreases toward the eastern part (Linares et al., 2011) such that high mean precipitation values occur in the
485 westernmost population of Grazalema, as compared to the easternmost population of Saucillo. This longitudinal
486 differentiation was also reflected in the demographic history inferred by DIYABC using nSSR and cpSSR
487 markers (Figure 4) indicating that potential adaptive divergence of easternmost and westernmost populations
488 should be subject to further research. Similar spatial differentiation has been suggested for *A. cilicica* in
489 Lebanon, where it grows as remnant populations (Awad et al., 2014), although, contrasting to this research, we
490 did not detect significant genetic differentiation related to elevation.

491 The different cohorts studied here (old, mature and young trees) did not show statistically significant
492 differences in genetic diversity. Thus we conclude that, at least under the time scale investigated here, the
493 populations are not undergoing genetic erosion. Studies reporting significant genetic erosion among cohorts of
494 trees species are very scarce. For instance, the genetic diversity obtained across diameter classes, used as a
495 surrogate for age classes, of the relict spruce *P. chihuahuana* decreased significantly in only one very small
496 population (Wehenkel and Saenz-Romero 2012). Thus, the current distribution *A. pinsapo* in south Spain
497 appears to be the result of long-term range retraction and local persistence as marginal populations (Terrab et
498 al., 2007; Linares 2011; Dering et al., 2014; Sanchez-Robles et al., 2014).

499

500 4.3. Concluding remarks and conservation insights

501 Our results support relatively high levels of genetic diversity in this species. Conservation actions are
502 generally based on adaptive genetic variation, which often does not match with neutral molecular variation. It
503 must be stressed that most variation among the *A. pinsapo* populations, reported here and by previous studies,
504 might be likely caused by genetic drift. While drift is not expected to routinely affect fitness, it can lead to the
505 fixation of some alleles and the loss of others (Hampe and Petit 2005; Hampe and Jump 2011). Under a drier
506 and warmed climate, the genetic diversity observed in this relict and drought-sensitive fir would be subjected to
507 selective pressure, no matter if this genetic diversity was essentially determined by random genetic drift. Hence,
508 the coming patterns will likely differs from the neutral expectations and they may provide unexpected adaptive
509 consequences (Kuparinen et al., 2010; Alberto et al., 2013). Knowledge on adaptive genetic variation in *A.*
510 *pinsapo* is still lacking, while significant phenotypic plasticity regarding carbon and water balance responses to
511 local climate suggests putative adaptive capacity in this relict fir (Lechuga et al., 2019 and references therein).
512 Hence, further research is necessary to assess the putative loss of evolutionary potential in these stands as well
513 as to identify divergence patterns of adaptive relevance.

514 The genetic differentiation of some populations, particularly Saucillo and Grazalema, may guide further
515 research focused on adaptive evolutionary processes, such as epigenetic mechanisms or phenotypic traits
516 related to drought tolerance (Neale and Wheeler 2019). Such research should also include the conservation
517 status and management of the closely related North-African populations of *A. marocana* and *A. tazaotana*
518 (Terrab et al., 2007; Jaramillo-Correa et al., 2010; Dering et al., 2014; Sanchez-Robles et al., 2014).
519 Conservation efforts should focus on reducing the probability of stochastic events, such as fires together with
520 preventing further habitat loss due to human impacts or climate change, while ex-situ conservation of genetic
521 resources or assisted migration would be also valuable given the limited migration potential of the species due
522 to topographic and landscape constraints. Furthermore, managing stand structure to reduce competition
523 provides a promising strategy to reduce climate change risks on some drought-sensitive tree species (Lechuga et
524 al 2017, 2019). Weak competitive ability has already been stated, for instance, in relict yew (*Taxus baccata* L.)
525 populations (Iszkuło et al 2012), while removal of competing vegetation has been recommended as adaptive
526 management in other relict conifers (Wehenkel and Saenz-Romero 2012).

527 **Author Contributions:** I. Cobo-Simón wrote the manuscript. J. Gallego and J. C. Linares conceived the idea. J.
528 C. Linares performed the field sampling. I. Cobo-Simón, J. Seco, and B. Mendez-Cea performed the laboratory
529 analysis. I. Cobo-Simón carried out the statistical analyses. I. Cobo-Simón, J. Gallego, J. C. Linares and A.

530 Jump conducted the statistical analyses results discussion. All authors contributed to the final writing of the
531 manuscript.

532 **Funding:** I. Cobo-Simón was supported by a Predoctoral grant BES-2014-070379, Spanish Ministry of
533 Economy. This study was supported by project CGL2013-48843-C2-2-R, Spanish Ministry of Economy.

534 **Acknowledgments:** We thank José Antonio Carreira de la Fuente and Noelia González Muñoz for their support
535 during fieldwork.

536 **Conflicts of Interest:** The authors declare no conflict of interest.

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- 730

731 **Supplementary material**732 *Appendix 1. DNA Extraction, Microsatellite and Inter-Microsatellite Genotyping*

733 Total genomic DNA was successfully extracted and purified from 100 mg of leaves per sample using the
734 QIAGEN DNeasy plant mini kit according to manufacturer's protocol (Pérez-González et al., 2018). Quality
735 and quantity of the DNA extraction was measured by means of 1% agarose gel and Nanodrop, respectively. We
736 used 8 nuclear microsatellites to perform genomic DNA amplification: NFF2, NFF3, NFH15, NFH3 and NFF7
737 developed for *A. nordmanniana* Stev. (Hansen et al., 2005); and Pin8, Pin20 and Pin48 developed for *A.*
738 *pinsapo* (Sánchez-Robles et al., 2012). In addition, we amplified 3 chloroplastic microsatellites: Pt30204,
739 Pt71936 and Pt15169 developed for *Pinus thunbergii* (Vendramin et al., 1996). Microsatellite selection was
740 done based on previous studies that prove that they yield enough polymorphic bands in other species
741 phylogenetically related with *A. pinsapo*. 4 fluorescent dyes were used to label forward primers on their 5' end:
742 FAM (blue), VIC (green), PET (yellow) or NED (red) (Eurofins MWG Operon). Then, all individuals were
743 amplified by polymerase chain reaction (PCR). The PCR mix contained 5 microlitres of DNA AmpliTools
744 Master Mix 2x (Biotools), 1 microlitres of each primer, forward and reverse (5 mM), 1 microlitre of
745 fluorescent dye, 0.5 microlitre of template DNA (30 ng) and 1.5 microlitres of autoclaved miliQ purified water
746 to obtain a total volume of 10 microlitres for each sample. The thermal cycling consisted of an initial
747 denaturation step at 94°C for 3 min, 3-step cycling repeated 35 times and consisting of denaturation at 94° for 1
748 minute, annealing at 56° for 1 minute and extension at 72° for 80 seconds; and a final extension step at 72° for 8
749 minutes (Pérez-González et al., 2018).

750 ABI 3730XL automated sequencer (Applied Biosystems) with the GeneScan™ - 500 LIZ™ size standard
751 (Applied Biosystems) was used to perform a capillary electrophoresis with the obtained PCR products. Allelic
752 binning and scoring of genotypes were carried out manually by two different people using the software
753 GeneMapper 4.1 (Applied Biosystems) and compared to get the final data set, with the objective of reducing the
754 possibilities of genotyping mistakes related to automated or arbitrary decisions in allelic binning (Amos et al.,
755 2007). 19 ISSR primers from the University of British Columbia, Canada (UBC) were tested in two individuals
756 to select those that yield more polymorphic bands. They were amplified by polymerase chain reaction (PCR).
757 The PCR mix contained 5 microlitres of DNA AmpliTool Master Mix 2x (Biotools), 2 microlitres of primer (5
758 mM), 0.5 microlitres of template DNA (30 ng) and 2.5 microlitres of autoclaved miliQ purified water to give a
759 total volume of 10 microlitres for each sample. The thermal cycling consisted of an initial denaturation step at

760 94°C for 5 min, 3-step cycling repeated 35 times and consisting of denaturation at 94° for 30 seconds, annealing
761 at 52° for 45 seconds and extension at 72° for 2 minutes; and a final extension step at 72° for 6 minutes.

762 The PCR products were analysed using a multicapillary electrophoresis system with a modified AL420
763 method file (QIAxcel DNA High Resolution Kit). Two replicates of the PCR products were made to evaluate
764 the consistency of the bands obtained. ISSR band outputs were counted automatically using the QIAxcel Bio
765 Calculator with thresholds for similarity set a baseline filter = 100 rfu, threshold = 15 %, minimum distance =
766 2.00 bp. Each sample profile was tested visually to eliminate miscalled or poorly identified peaks. Then, those
767 bands that were not found in both replicates of each individual were removed. Thus, we ensure the repeatability
768 of the bands. The QIAxcel Bio Calculator was used to produce a presence/absence binary score for each sample.
769 For each primer, amplified fragments with the same molecular weight (bp) were documented as present (1) or
770 absent (0). We used the obtained binary matrix in the further analyses. We accepted that each band showed one
771 Mendelian locus with two alleles, the ‘dominant’ or visible alleles and the ‘recessive’ or null alleles. We also
772 accepted that alleles from different loci do not migrate at the same position.

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787 **Table S1.** Null alleles frequency estimated by Expectation Maximization (EM) algorithm in nSSR loci.
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		Null alleles estimated frequency
Locus	NFF2	0.052
	NFF3	0.044
	NFH15	0.024
	NFH3	0.044
	NFF7	0.044
	Pin8	0.034
	Pin20	0.074
	Pin48	0.084
Population	S	0.086
	C	0.031
	A	0.024
	G	0.013
	P	0.101

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793 **Table S2.** Hierarchical AMOVA based on ISSR markers for different levels of analysis (among
 794 populations, among elevation cohorts and among age cohorts). *Statistically significant p-values.

795

ISSR	Level of analysis	df	SS	MS	Est. Var.	%	PhiTP	P-value
5 pops	Among Pops	5	256.334	51.267	1.386	12%	0.120	0.001*
	Within Pops	194	1969.331	10.151	10.151	88%		
	Total	199	2225.665		11.537	100%		
Saucillo by elevation	Among Pops	2	1.149	0.574	0.000	0%	-0.048	0.957
	Within Pops	39	55.604	1.426	1.426	100%		
	Total	41	56.753		1.426	100%		
Caucon by elevation	Among Pops	2	2.354	1.177	0.000	0%	-0.020	0.894
	Within Pops	69	141.877	2.056	2.056	100%		
	Total	71	144.231		2.056	100%		
Grazalema by elevation	Among Pops	2	7.151	3.576	0.316	16%	0.159	0.003*
	Within Pops	15	25.167	1.678	1.678	84%		
	Total	17	32.318		1.994	100%		
Saucillo by age	Among Pops	3	154.786	51.595	3.743	22%	0.222	0.002*
	Within Pops	38	497.500	13.092	13.092	78%		
	Total	41	652.286		16.835	100%		
Caucon by age	Among Pops	2	4.787	2.394	0.016	1%	0.008	0.248
	Within Pops	69	139.498	2.022	2.022	99%		
	Total	71	144.285		2.037	100%		
Animas by age	Among Pops	2	17.314	8.657	0.400	16%	0.162	0.001*
	Within Pops	48	99.118	2.065	2.065	84%		
	Total	50	116.433		2.465	100%		

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797

798 **Table S3.** Hierarchical AMOVA based on cpSSR markers for different levels of analysis (among
 799 populations, among elevation cohorts and among age cohorts) *Statistically significant p-values.
 800

cpSSR	Level of analysis	df	SS	MS	Est. Var.	%	PhiPT	P-value
5 pops	Among Pops	4	335.228	83.807	1.655	5%	0.041	0.038*
	Within Pops	184	6186.084	33.620	33.620	95%		
	Total	188	6521.312		35.075	100%		
Saucillo elevation	Among Pops	2	73.424	36.712	1.104	5%	0.046	0.143
	Within Pops	37	844.726	22.830	22.830	95%		
	Total	39	918.150		23.934	100%		
Caucon elevation	Among Pops	2	6.429	3.215	0.000	0%	-0.045	0.962
	Within Pops	67	2310.328	34.483	34.483	100%		
	Total	69	2316.757		34.483	100%		
Grazalema elevation	Among Pops	2	230.667	115.333	11.992	16%	0.158	0.167
	Within Pops	11	703.333	63.939	63.939	84%		
	Total	13	934.000		75.931	100%		
Saucillo by age	Among Pops	3	40.150	13.383	0.000	0%	-0.049	0.735
	Within Pops	36	878.000	24.389	24.389	100%		
	Total	39	918.150		24.389	100%		
Caucon by age	Among Pops	2	40.014	20.007	0.000	0%	-0.018	0.613
	Within Pops	67	2276.743	33.981	33.981	100%		
	Total	69	2316.757		33.981	100%		
Animas by age	Among Pops	2	11.320	5.660	0.000	0%	-0.055	0.948
	Within Pops	47	1609.100	34.236	34.236	100%		
	Total	49	1620.420		34.236	100%		

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803 **Table S4.** Hierarchical AMOVA based on nSSR markers for different levels of analysis (among
 804 populations, among elevation cohorts and among age cohorts).

nSSR	Level of analysis	df	SS	MS	Est. Var.	%	Fst	P-value
5 pops	Among Pops	5	61.017	12.203	0.170	7%	0.065	0.001*
	Among Indiv	185	495.166	2.677	0.257	10%		
	Within Indiv	191	413.036	2.162	2.162	84%		
	Total	381	969.220		2.589	100%		
Saucillo elevation	by Among Pops	2	8.321	4.161	0.049	2%	0.020	0.017*
	Among Indiv	37	108.673	2.937	0.603	25%		
	Within Indiv	40	69.253	1.731	1.731	73%		
	Total	79	186.247		2.383	100%		
Caucon elevation	by Among Pops	2	7.701	3.850	0.029	1%	0.012	0.038*
	Among Indiv	67	176.421	2.633	0.199	8%		
	Within Indiv	70	156.500	2.236	2.236	91%		
	Total	139	340.621		2.463	100%		
Grazalema elevation	by Among Pops	2	3.095	1.548	0.000	0%	-0.026	0.742
	Among Indiv	11	22.333	2.030	0.000	0%		
	Within Indiv	14	33.500	2.393	2.393	100%		
	Total	27	58.929		2.393	100%		
Saucillo by age	Among Pops	3	8.294	2.765	0.000	0%	-0.006	0.684
	Among Indiv	36	108.640	3.018	0.643	27%		
	Within Indiv	40	69.269	1.732	1.732	73%		
	Total	79	186.204		2.375	100%		
Caucon by age	Among Pops	2	4.154	2.077	0.000	0%	-0.005	0.871
	Among Indiv	67	179.967	2.686	0.225	9%		
	Within Indiv	70	156.500	2.236	2.236	91%		
	Total	139	340.621		2.461	100%		
Animas by age	Among Pops	2	7.110	3.555	0.034	1%	0.014	0.065
	Among Indiv	47	116.610	2.481	0.053	2%		
	Within Indiv	50	118.791	2.376	2.376	96%		
	Total	99	242.511		2.462	100%		

806 *Appendix 2. Evolutionary scenarios tested with DIYABC 2.1.0.*

807 Seven evolutionary scenarios were tested with DIYABC 2.1.0 based on the results obtained with
808 the different parameters used to test genetic differentiation among populations and assuming
809 hypothetical divergence times (t_1, t_2, \dots, t_n): (i) Grazalema and Sierra de las Nieves populations
810 diverged from an ancestral population at t_1 , followed by Saucillo at t_2 and then the rest of the
811 studied populations (Caucon, Animas and Pilonés) diverged simultaneously at t_3 ; (ii) Saucillo and
812 the rest of the populations diverged from an ancestral population at t_1 , followed by Grazalema at t_2
813 and then the rest of the studied populations (Caucon, Animas and Pilonés) diverged simultaneously
814 at t_3 ; (iii) Grazalema and the rest of the studied populations diverged from an ancestral population
815 at t_2 , which split at the same time at time t_3 ; (iv) Saucillo and the rest of the populations diverged
816 from an ancestral population at t_2 , which split at the same time at time t_3 . These four scenarios were
817 based on the fact that Saucillo and Grazalema constituted the most different populations based on
818 the previous analyses. (v) Split from west: Grazalema and the rest of populations diverged from an
819 ancestral population at t_1 , followed by Pilonés at t_2 , Animas at t_3 , and Caucon and Saucillo at t_4 ;
820 (vi) Split from east: Saucillo and the rest of populations diverged from an ancestral population at t_1 ,
821 followed by Caucon at t_2 , Animas at t_3 , and Pilonés and Grazalema at t_4 ; (vii) split at the same
822 time: all populations diverged from an ancestral population at time t_1 . All these scenarios were
823 replicated to study the presence of bottlenecks, in order to test the results previously obtained by
824 BOTTLENECK.
825

826 **Table S5.** Prior distributions of the parameters used in DIYABC analyses.

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Parameter	Minimum	Maximum
Effective population size	1	10000
Time scale in generations	1	10000
Mutation model		
Mean mutation rate	1×10^{-4}	1×10^{-3}
Individual locus mutation rate	1×10^{-5}	1×10^{-2}
Mean coefficient P	1×10^{-1}	3×10^{-1}
Individual locus coefficient P	1×10^{-2}	9×10^{-1}
Mean SNI rate	1×10^{-8}	1×10^{-4}
Individual locus SNI rate	1×10^{-9}	1×10^{-3}

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831 **Table S6.** Median values of effective population sizes estimated for the different ancestors,
 832 populations prior to simulated bottlenecks and current populations (see Table 1 for abbreviations).
 833 Divergence times (number of generations) were obtained by DIYABC using nSSR, cpSRR, and
 834 both nSSR and cpSSR molecular markers together. Generation time was assumed 20 years for the
 835 recalculation of historical time of divergence (Time BP 1, Time BP 2, Time BP 3, BP: years before
 836 present).

837

	nSSR	cpSSR	nSSR + cpSSR
Ancestor 1	5804	7120	7157
Ancestor 1	4192	4032	5371
S	3120	2695	2437
C	1647	3282	3157
A	3518	4696	2913
P	3789	2219	3208
G	3852	1178	2959
S bottleneck	7915	7199	5984
C bottleneck	6150	6754	6882
A bottleneck	6490	6063	6914
P bottleneck	8263	6976	6401
G bottleneck	6166	6108	7352
Divergence 1	5446	6882	5401
Divergence 2	2677	2670	3455
Divergence 3	2145	2657	2099
Time BP 1	108920	137640	108020
Time BP 2	53340	53400	69100
Time BP 3	42900	53140	41980

838

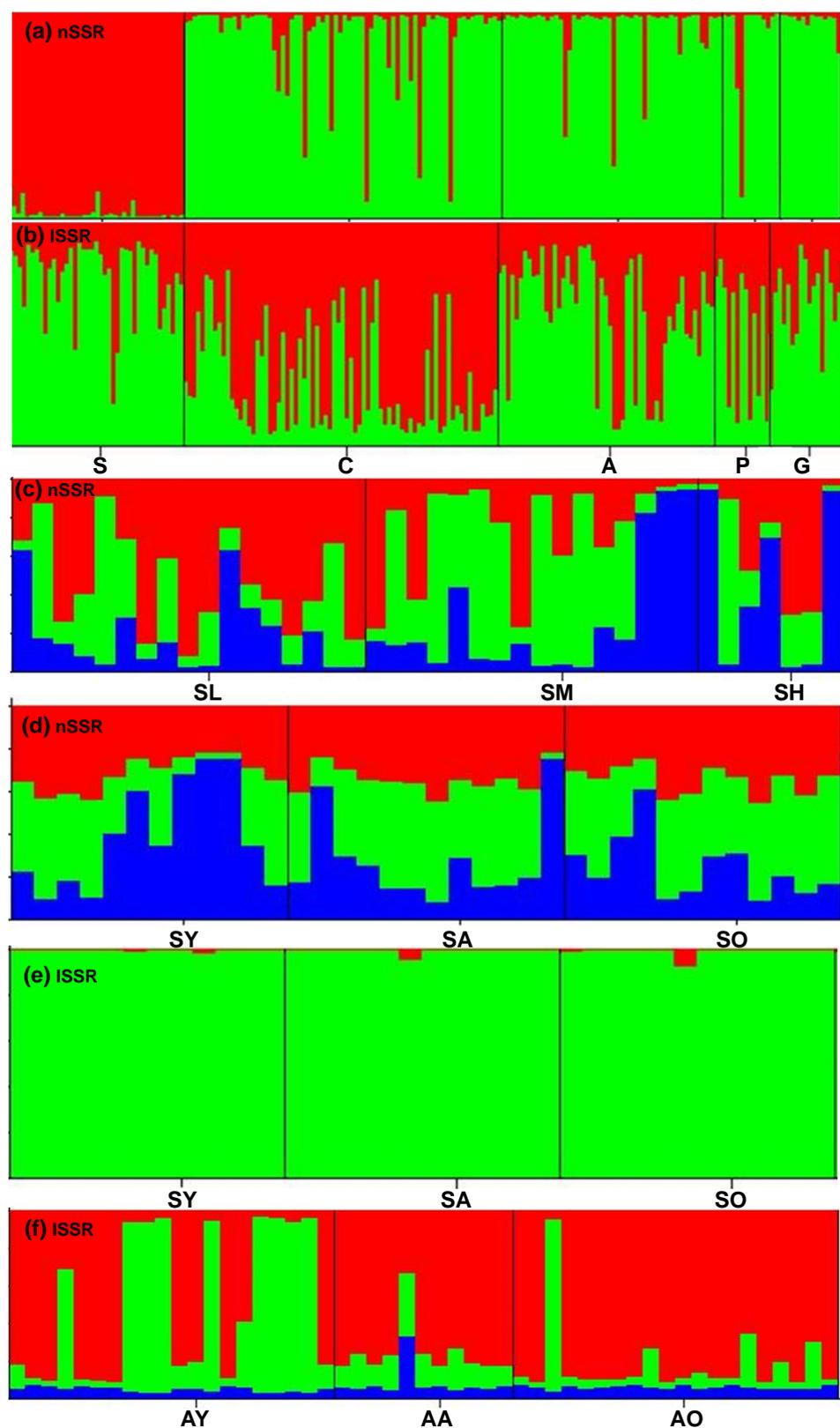


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840 **Figure S1.** PCoA analyses based on pairwise Nei's standard genetic distances sorted by elevations
 841 and based on nSSR (a), and ISSR (b); and sorted by age cohorts and based on nSSR markers (c).

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845 **Figure S2.** Proportion of the membership coefficient for each individual in six *Abies pinsapo* forests based
 846 on nSSR (a) and ISSR (b); Saucillo population based on nSSR sorted by elevation (c) and age (d), and based
 847 on ISSR sorted by age (e); Animas population based on ISSR sorted by age (f). Inferred clusters used $K = 2$
 848 (a, b and e) and $K = 3$ (c, d and f) in STRUCTURE analysis. Only results showing disconnected populations
 849 are shown (see codes in Table 1).